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Organic Solvent Tolerance and Antibiotic Resistance Increased by Overexpression of *marA* in *Escherichia coli*

HIROYUKI ASAKO, HARUSHI NAKAJIMA,* KEI KOBAYASHI, MASATO KOBAYASHI, AND RIKIZO AONO

Department of Bioengineering, Tokyo Institute of Technology, 4259 Nagatsuta, Yokohama 226, Japan

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We previously reported that overexpression of the *soxS* or *robA* gene causes in several *Escherichia coli* strains the acquisition of higher organic solvent tolerance and also increased resistance to a number of antibiotics (H. Nakajima, K. Kobayashi, M. Kobayashi, H. Asako, and R. Aono, Appl. Environ. Microbiol. 61:2302–2307, 1995). Most *E. coli* strains cannot grow in the presence of cyclohexane. We isolated the *marRAB* genes from a Kohara λ phage clone and cyclohexane-tolerant mutant strain OST3408. We found a substitution of serine for arginine at position 73 in the coding region of *marR* of OST3408 and designated the gene *marR08*. Our genetic analysis revealed that *marR08* is responsible for the cyclohexane-tolerant phenotype. We observed that the *marA* gene on high-copy-number plasmids increased the organic solvent tolerance of *E. coli* strains. Furthermore, exposure of *E. coli* cells to salicylate, which activates the *mar* regulon genes, also raised organic solvent tolerance. Overexpression of the *marA*, *soxS*, or *robA* gene increased resistance to numerous antibiotics but not to hydrophilic aminoglycosides.

Most water-immiscible organic solvents are generally toxic to microorganisms. In a nonionizable organic solvent-aqueous two-phase culture, toxicity of an organic solvent is inversely correlated with the log of the partition in *n*-octanol–water ($\log P_{ow}$ value) (12) of the solvent (18, 19). An organic solvent with a low $\log P_{ow}$ value is generally toxic to most microorganisms. Organic solvent tolerance levels of microorganisms significantly differ with species. For example, while *Escherichia coli* IFO3806 grows in the presence of organic solvents which have greater $\log P_{ow}$ values than propylbenzene ($\log P_{ow}$, 3.8), *Pseudomonas putida* IFO3738 grows even in the presence of *p*-xylene ($\log P_{ow}$, 3.1) (18). In recent years, highly organic solvent-tolerant microorganisms, which thrive in the presence of toluene ($\log P_{ow}$, 2.7), were isolated from forest or garden soil and identified as *P. putida*, *Pseudomonas aeruginosa*, and *Pseudomonas fluorescens* (2, 18, 31). *E. coli* JA300 grows in the presence of *n*-hexane ($\log P_{ow}$, 3.9) but not in the presence of cyclohexane ($\log P_{ow}$, 3.4) (1). We isolated a series of organic solvent-tolerant mutants from strain JA300. One of the mutants, OST3408, shows growth in the presence of cyclohexane, and the most tolerant mutant, OST3121, can grow in the presence of *p*-xylene (1). We previously reported that the organic solvent tolerance levels of *E. coli* strains can be improved by overexpression of the stress response genes *soxS* and *robA* (32, 33). The *soxS* or *robA* gene on high-copy-number plasmids under control of the *lac* promoter confers cyclohexane tolerance to JA300. Overexpression of the *robA* gene also raises resistance to a number of antibiotics and heavy metal ions. The spectrum of resistance to antibiotics is similar to that of the *soxS*-overexpressing strain (33). A number of spontaneously isolated cyclohexane-tolerant mutants of JA300 simultaneously acquired resistance to low levels of ampicillin, chloramphenicol, nalidixic acid, and tetracycline but became more sensitive to kanamycin than JA300 (4).

Resistance to a number of structurally unrelated antibiotics can be induced in *E. coli* by derepression of the *marRAB* operon. The *marRAB* region at 34 min on the *E. coli* chromosome has been cloned and sequenced (10), and its regulation has been studied. The *marRAB* operon is negatively autoregulated by the MarR protein, which binds to the *marRAB* promoter region in vitro (27). Treatment with certain antibiotics or aromatic weak acids such as salicylate or acetylsalicylate derepresses this operon (11, 16, 28). The MarA protein is a member of the AraC subfamily of helix-turn-helix transcriptional activators and has more than 45% sequence identity with the SoxS and Rob proteins (10, 14, 15). The MarA protein controls a set of genes (*mar* and *soxRS* regulons, including *sodA*, *nfo*, *micF*, *inaA*, *fumC*, *zwf*, and *fpr*) and provides *E. coli* cells with resistance to a large number of antibiotics and superoxide-generating reagents (5, 9, 21, 25, 34, 36).

We report here the acquisition of higher organic solvent tolerance in several *E. coli* strains by the high-copy-number *marA* gene or by exposure to salicylate. We identify *marR08* mutation, which is responsible for the cyclohexane-tolerant phenotype of OST3408 and its derivatives. Overexpression of the *marA*, *soxS*, or *robA* gene also increases the resistance to numerous hydrophobic antibiotics but not to highly hydrophilic aminoglycosides.

MATERIALS AND METHODS

Bacterial strains and plasmids. The *E. coli* strains used in this study are listed in Table 1. Charomid 9-28 (a cosmid vector for cloning a 10- to 24-kb DNA fragment) was purchased from Nippon Gene Co. (Tokyo, Japan) (35). pBluescript II (pBSII) KS^+ and SK^+ vectors were purchased from Toyobo Biochemical, Inc. (Osaka, Japan). pHc3R is pBSII carrying the *soxS* gene on a 0.4-kb *Hin*cII fragment under the control of the *lac* promoter (32). pOST41BR is pBSII carrying the *robA* gene on a 1.9-kb *Sal*I-*Bam*HI fragment under the control of the *lac* promoter (33). λ305 (2H2) is a Kohara λ phage clone which spans the min 34 region on the *E. coli* chromosome (23). Plasmid pHA102 is pBSII SK^+ carrying the *marRAB* genes on a 2.0-kb *Sca*I-*Cla*I fragment derived from λ305. pHA103, pHA104, pHA105, pHA106, and pHA107 are deletion derivatives from pHA102 containing the *marAB*, *marR*, *marA*, *marB*, and *marRA* genes, respectively (see Fig. 1a).

Media and chemicals. *E. coli* cells were grown in modified Luria broth (LBG medium; pH was adjusted to 7.0) consisting of 1% (wt/vol) Bacto Tryptone (Difco Laboratories), 0.5% Bacto Yeast Extract (Difco), 1% NaCl, and 0.1% glucose at 37°C. In organic solvent tolerance assays, $MgSO_4$ was added (10 mM) to the LBG medium (LBGMg medium) to stabilize the viability of *E. coli* cells in

* Corresponding author. Mailing address: Department of Bioengineering, Tokyo Institute of Technology, 4259 Nagatsuta, Yokohama 226, Japan. Fax: (81) 45-924-5819. E-mail: hanakaji@bio.titech.ac.jp.

TABLE 1. Bacterial strains used in this study

Strain	Genotype	Reference
W3110	λ⁻ *rrnD rrnE*	23
JA300	F⁻ *leuB trpC thr lac thi rpsL hsdS*	22
OST3408	F⁻ *leuB trpC thr lac thi rpsL hsdS marR08*	1
OST3408Tc	F⁻ *leuB trpC thr lac thi rpsL hsdS marR08 zde-234*::Tn*10*	This study
OST4251	F⁻ *trpC thr lac thi rpsL hsdS araD ksgA ostA*	3
MC1061	*hsdR araD* Δ(*araABC-leu*) *lac galU galK rpsL thi*	8
DH1	*supE hsdR recA endA gyrA thi relA*	17
FS1576	*supE hsdR thi leuB lacY recD*	38
PLK1110	*zde-234*::Tn*10 argA pheA trp supE rpsL*	7

the presence of solvents (19). Organic solvents used for this study, diphenyl ether, *n*-hexane, cyclohexane, and *p*-xylene, were purchased from Wako Pure Chemical Industries (Osaka, Japan). Chloramphenicol, enoxacin, erythromycin, gentamicin, kanamycin, kasugamycin, nalidixic acid, neomycin, norfloxacin, novobiocin, ofloxacin, and phosphomycin (Sigma Chemical Co., Rahway, N.J.) and tetracycline hydrochloride (Wako Pure Chemical) were used for the antibiotic resistance assay. The logP_{ow} values of antibiotics were calculated by the ClogP program (Adam Net Co., Tokyo, Japan) (24).

Organic solvent tolerance assay. (i) Liquid-medium assay. A 100-μl culture of overnight-grown *E. coli* W3110 cells carrying pHA104 (*marR*) or pHA105 (*marA*) was inoculated to 10 ml of fresh LBGMg medium and incubated at 37°C. At the early exponential phase of growth, the culture was overlaid with a 10% volume of cyclohexane and incubated at 37°C with shaking. Growth was monitored by measuring turbidity (optical density at 660 nm) and by counting the number of viable cells in the aqueous layer.

(ii) Solid-medium assay. Freshly grown *E. coli* cells were suspended in 0.9% NaCl (approximately 10^7 cells per ml), and a drop of the suspension (5 μl) was spotted on a solid LBGMg medium. The surface of the medium was overlaid with an organic solvent to a thickness of 3 mm. The plates were sealed and incubated at 37°C for 14 h.

In order to examine the effect of salicylate on organic solvent tolerance, colony-forming efficiency was measured. W3110 cells carrying pBSII or pHA105 were grown in LBGMg medium (pH 7.0). At early stationary phase, the culture was diluted 20-fold with fresh LBGMg medium (5 ml) containing 2 mM sodium salicylate and incubated at 30°C for 100 min. When the cell turbidity (optical density at 660 nm) reached 0.4, serial 10-fold dilutions of the cultures were prepared and 5 μl of each suspension was spotted onto solid LBGMg medium containing 2 mM sodium salicylate. Approximately 10^5, 10^4, 10^3, 10^2, and 10 cells were contained in the spots. The medium was overlaid with cyclohexane and incubated at 37°C for 16 h.

Antibiotic susceptibility. The MICs of various antibiotics were determined by a sequential dilution method (33). LBG medium liquid cultures containing different concentrations of antibiotic and freshly grown 10^3 cells of the tested *E. coli* strain were incubated at 37°C for 18 h. The lowest concentration of antibiotic which completely inhibited growth was defined as the MIC (15).

Genetic analysis. DNA manipulations, including preparation of *E. coli* chromosomal DNA, plasmid DNA preparations, restriction enzyme digestion and ligation, and transformation of *E. coli*, were carried out by standard methods (26). Chromosome DNA fragments of *E. coli* OST3408 were ligated with Charomid 9-28 and infected into JA300 cells by use of an in vitro packaging kit (LAMBDA INN; Nippon Gene Co.). Synthetic DNA oligonucleotides were purchased from Biologica Co. (Nagoya, Japan). The *marRAB* region of OST3408 was amplified from the chromosome DNA by the TaKaRa EX Taq PCR method (TakaraShuzo Co. Ltd., Kyoto, Japan) using GeneAmp PCR System 2400 (Perkin-Elmer Applied Biosystems, Foster City, Calif.). Nucleotide sequences of the cloned DNA fragments were determined with a DNA sequencing system (model 373A; Perkin-Elmer Applied Biosystems). The DNA sequence data were analyzed with the BioDatabase CD-ROM GENETYX-MAC/CD (Software Development Co., Tokyo, Japan).

Generalized transduction was done with phage P1*kc* by the method described by Miller (30).

RESULTS

Cloning of the *mar* operon genes. Chromosomal DNA of *E. coli* OST3408 (cyclohexane tolerant [CH^r]) was prepared and partially digested with *Kpn*I. The DNA fragments were inserted into cosmid vector Charomid 9-28 and were introduced into JA300 (cyclohexane-sensitive [CH^s]) cells. From 3.3×10^4 ampicillin-resistant transformants, nine CH^r colonies were selected. Plasmid pE1, containing a 20-kb DNA insert, was recovered from one of the colonies. Subclone analysis of the insert DNA on high-copy-number vector pBSII revealed that one of the plasmids containing a 3.0-kb fragment (designated pOST482) provided cyclohexane tolerance to JA300.

The partially determined DNA sequence and restriction enzyme map showed that the 3.0-kb fragment contained the 0.7-kb *marRA* locus, lacking the codons for the N-terminal 25 amino acids of MarR and the codons for the C-terminal 16 amino acids of MarA (designated *mar′RA′*). We obtained a complete length of the *marRAB* gene from the Kohara phage library 2H2 clone (23). A 2.0-kb *Sca*I-*Cla*I DNA fragment containing the *marRAB* region was subcloned into pBSII. The resultant plasmid, pHA102, was utilized for the following subcloning experiments (Fig. 1a).

The *marR08* mutation is responsible for the cyclohexane-tolerant phenotype of OST3408. We designed a sense primer (5′-TCGCTATGGTTCCCGGACCGGC-3′) and an antisense primer (5′-GCAACCATGATTCACAGTCTGGTTA-3′) based on the 5′ and 3′ flanking sequences of the *marRAB* operon, respectively. In order to isolate the full-length *marRAB* operon from OST3408, PCR was performed using these synthesized oligonucleotides as primers and chromosome DNA of OST3408 as a template. From the resultant PCR product, the 1.9-kb *Sca*I-*Cla*I DNA fragment containing the *marRAB* region was cloned into pBSII. DNA sequence analysis showed that the *marR* gene derived from OST3408 has one novel point mutation, a C → A transition at codon 73 (Arg → Ser) (Fig. 1b). We defined this mutant *marR* gene as *marR08*. We investigated whether the *marR08* mutation is involved in the cyclohexane-tolerant phenotype. Transposon *zde-234*::Tn*10* (Tc^r) of *E. coli* PLK1110 is 0.4 min distant from the *mar* locus on the *E. coli* chromosome map. P1*kc* phage grown on PLK1110 (Tc^r CH^s) was used to infect the recipient, OST3408 (Tc^s CH^r). From 21 Tc^r transductants, 11 strains showed cyclohexane tolerance (CH^r) and the others lost the tolerance (CH^s). One of the Tc^r CH^r strains, designated OST3408Tc, was used for further analysis. P1*kc* phage grown on OST3408Tc was used to infect Tc^s-, CH^s-recipient strains JA300, OST4251, MC1061, and FS1576. From the selected Tc^r transductants, 12% (6 of 50; JA300), 11% (2 of 18; OST4251), 9% (5 of 55; FS1576), and 18% (18 of 100; MC1061) of the strains showed the CH^r phenotype. OST3408 and OST3408Tc were resistant to two- to fourfold higher concentrations of ampicillin, chloramphenicol, and nalidixic acid compared with JA300 and CH^s transduction derivatives. We isolated the *mar* locus DNA fragment from one of the Tc^r CH^r transductants derived from JA300. Sequence analysis revealed that the CH^r strain contained the *marR08* mutation. These results indicate that the *marR08* mutation is responsible for the cyclohexane-tolerant phenotype of OST3408 and its derivatives.

Effect of *marR* or *marA* gene expression on organic solvent tolerance. We constructed a series of subclones containing the *marAB*, *marR*, *marA*, *marB*, and *marRA* genes on high-copy-number vectors, designated pHA103, pHA104, pHA105, pHA106, and pHA107, respectively. Figure 1a shows the cyclohexane tolerance acquisition of JA300 cells carrying the *marA* gene under control of the *lac* promoter (pHA102, pHA103, pHA105, and pHA107). JA300 cells carrying the *marA* gene on a low-copy-number vector, pMW118, showed no growth in the presence of cyclohexane (pHA105L). These results indicate that overexpression of the *marA* gene increases organic solvent tolerance in *E. coli*. The high-copy-number *marB* gene (pHA106) had no effects on solvent tolerance. Interestingly, the *marR08* gene on high-copy-number vectors

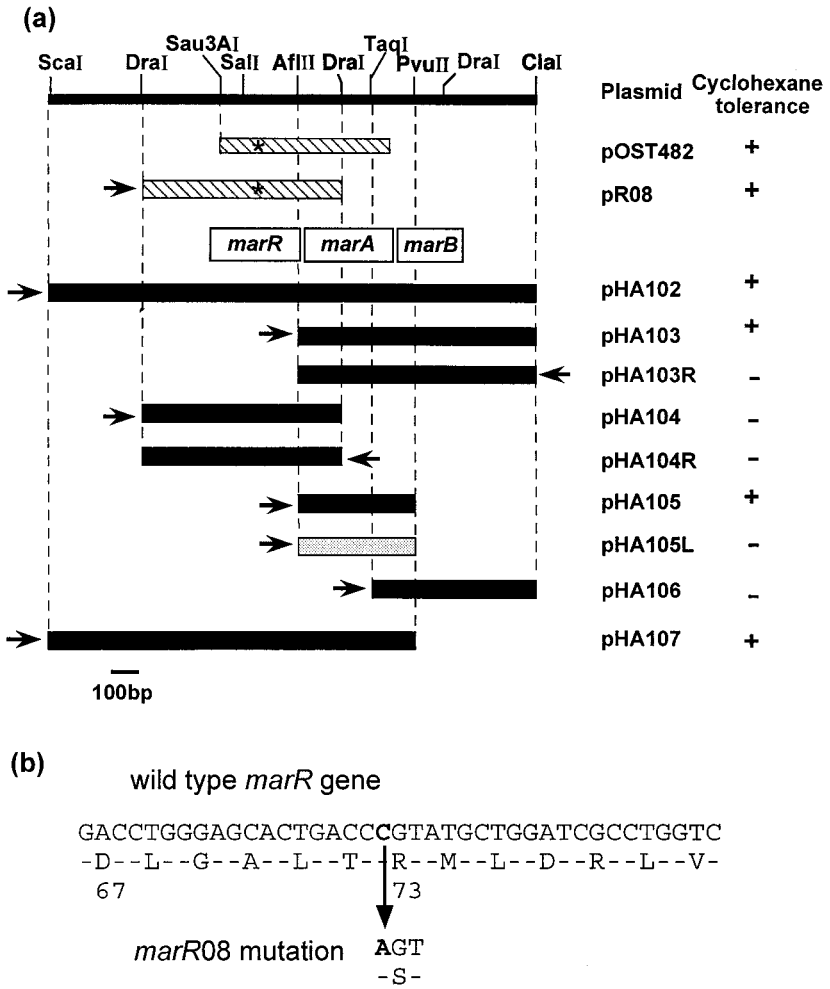

FIG. 1. (a) Physical map of the plasmids containing the *marRAB* region and conferring cyclohexane tolerance. Open bars represent open reading frames of the *marR*, *marA*, and *marB* genes. Solid bars represent subclones in pBSII; only insert DNAs are shown. The grey bar represents a subclone on low-copy-number vector pMW119. Striped bars represent the DNA fragments from OST3408, and asterisks indicate the *marR08* mutation. Arrows indicate the direction of the *lac* promoter on the vectors. Cyclohexane tolerance was tested by incubation of W3110 carrying each plasmid on LBGMg medium plates overlaid with cyclohexane; growth (+) or no growth (−) is indicated. (b) Nucleotide sequence change in the *marR08* mutation. The DNA region affected by the mutation is shown together with the regions from the 67th to the 79th amino acids of the MarR protein.

(pOST482 and pR08) raised the organic solvent tolerance of JA300.

Turbidity and viability of W3110 cells carrying the high-copy-number *marR* or *marA* gene in the presence of organic solvent were measured. When cyclohexane was added to W3110 culture (10% [vol/vol]) at the early exponential phase of growth (approximately 5×10^7 cells/ml), the increase in turbidity completely stopped and the number of viable cells decreased by 10^{-3} (Fig. 2). W3110 cells carrying pHA105 (*marA*) maintained slow growth even in the presence of cyclohexane. The number of viable cells carrying pHA105 stayed between 5×10^6 and 2×10^7/ml in 8 h. In contrast, the number of viable cells carrying pHA104 (*marR*) rapidly decreased to 10^2/ml by addition of cyclohexane (Fig. 2b). These growth characteristics indicate opposite functions of the *marA* and *marR* genes in susceptibility to organic solvents.

Overexpression of *marA* increases organic solvent tolerance in several *E. coli* strains. We investigated the effects of overexpression of the *marA* and *marR* genes on organic solvent tolerance of several *E. coli* strains. Table 2 shows that most of the tested strains carrying the high-copy-number *marA* plasmid grew in the presence of cyclohexane. *n*-Hexane-sensitive strain DH1 acquired *n*-hexane tolerance by pHA105. These results suggest that the high-copy-number *marA* gene generally raises organic solvent tolerance levels of *E. coli* strains. No additional increase in the organic solvent tolerance of strain OST3408 was observed. On the other hand, overexpression of the *marR* gene decreased organic solvent tolerance levels of *E. coli* strains.

Effect of salicylate on organic solvent tolerance. Exposure of *E. coli* cells to salicylate derepresses the *mar* operon and increases resistance to several antibiotics (11). We investigated organic solvent tolerance levels of *E. coli* cells exposed to salicylate. Freshly grown W3110 cells were provided for assay of colony-forming efficiency on LBGMg medium containing 2 mM sodium salicylate in the presence of cyclohexane. Although W3110 treated with salicylate formed two colonies from 10 cells, noninduced cells formed no colonies from 10^4 cells (Fig. 3). W3110 cells carrying the high-copy-number *marA* plasmid showed the same colony-forming efficiency (Fig. 3).

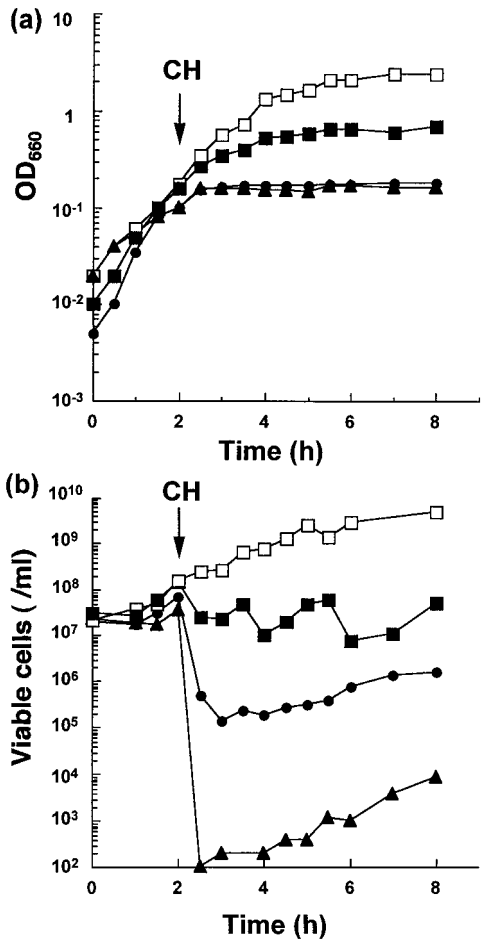

FIG. 2. Growth of *E. coli* W3110 carrying the *marR* or *marA* plasmid in the presence of cyclohexane. *E. coli* W3110 cells carrying pHA104 or pHA105 were incubated in 10 ml of LBGMg medium at 37°C, and at the time indicated with arrows, 1 ml of cyclohexane (CH) was added and incubated with shaking. (a) Growth of the cultures were monitored by measuring the turbidity (optical density at 660 nm [OD_{660}]). (b) Cultures were sampled periodically, and viable cells were counted on LBG medium plates. □, W3110, no organic solvent; ●, W3110(pBSII); ■, W3110 carrying high-copy-number *marA* (pHA105); ▲, W3110 carrying high-copy-number *marR* (pHA104).

Overexpression of the *marA*, *robA*, or *soxS* gene increases resistance to hydrophobic antibiotics. The MarA protein has strong homology with Rob and SoxS proteins. We previously reported the close correlation between organic solvent tolerance and antibiotic resistance (4), with kanamycin as an exception. We calculated logP_{ow} values of antibiotics from their structures and found that kanamycin (logP_{ow}, −7.77) is highly hydrophilic. MICs of antibiotics, including highly hydrophilic substances, were measured in W3110 cells carrying the high-copy-number *marA*, *robA*, or *soxS* plasmid. These stress response gene-activating cells showed increases in resistance to hydrophobic antibiotics, including novobiocin (logP_{ow}, 3.84), nalidixic acid (logP_{ow}, 1.57), chloramphenicol (logP_{ow}, 1.14), erythromycin (logP_{ow}, 0.65), ofloxacin (logP_{ow}, −0.10), phosphomycin (logP_{ow}, −0.26), norfloxacin (logP_{ow}, −0.54), enoxacin (logP_{ow}, −1.29), and tetracycline (logP_{ow}, −1.86), but not to highly hydrophilic antibiotics, including kasugamycin (logP_{ow}, −3.75), gentamicin (logP_{ow}, −4.08), kanamycin (logP_{ow}, −7.77), and neomycin (logP_{ow}, −9.03) (Table 3).

TABLE 2. Effect of high-copy-number *marA* and *marR* on organic solvent tolerance in several *E. coli* strains

Strain	Plasmid	Growth in the presence of indicated organic solvent (logP_{ow})[a]				
		DE (4.2)	H (3.9)	H-CH	CH (3.4)	pX (3.1)
W3110	pBSII	++	++	+	−	−
W3110	pHA105	++	++	++	++	−
W3110	pHA104	++	+	+	−	−
JA300	pBSII	++	++	−	−	−
JA300	pHA105	++	++	++	++	−
JA300	pHA104	++	+	−	−	−
MC1061	pBSII	++	++	−	−	−
MC1061	pHA105	++	++	++	++	−
MC1061	pHA104	++	+	−	−	−
DH1	pBSII	++	−	−	−	−
DH1	pHA105	++	++	++	−	−
DH1	pHA104	+	−	−	−	−
FS1576	pBSII	++	++	++	−	−
FS1576	pHA105	++	++	++	++	−
FS1576	pHA104	++	++	−	−	−
OST3408	pBSII	++	++	++	++	−
OST3408	pHA105	++	++	++	++	−
OST3408	pHA104	++	++	++	−	−

[a] A drop of *E. coli* cell suspension (5 μl) was spotted on a solid LBGMg medium and overlaid with an organic solvent. ++, luxuriant growth covered the entire surface of the spots; +, scattered colonies appeared in the spots; −, no growth; DE, diphenyl ether; H, *n*-hexane; H-CH, mixed solvent of *n*-hexane and cyclohexane (1:1, vol/vol); CH, cyclohexane; pX, *p*-xylene.

DISCUSSION

We isolated the *mar* operon region from an organic solvent-tolerant mutant *E. coli* strain, OST3408, and found a substitution mutation in the *marR08* gene coding region. The MarR protein binds to the *mar* operator sequences and negatively regulates expression of the *marRAB* operon (27). The *marA* gene on a high-copy-number plasmid increased the organic solvent tolerance of several *E. coli* strains. On the other hand, the high-copy-number wild-type *marR* plasmid but not the *marR08* plasmid decreased solvent tolerance. P1 transduction analysis revealed that the *mar* locus of the OST3408 strain is involved in the cyclohexane-tolerant phenotype.

Mutations in the *marR* locus generally derepress the *mar* operon. Several *marR* mutations (e.g., *marR1*, *soxQ1*, and *cfxB1*), which have pleiotropic effects on antibiotic resistance and expression of oxidative stress genes, have been reported elsewhere (5). The MarR protein shows homology to a group of similarly small proteins containing EmrR, PecS, and HpcR, which act as negative regulators (37). The mutations in *soxQ1* (A70T), *marR1* (R77L) and *marR08* (R73S) are located at the

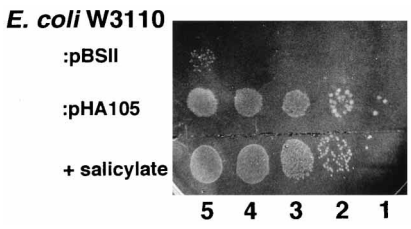

FIG. 3. Effect of salicylate on organic solvent tolerance. Colony-forming efficiency of *E. coli* W3110 in the presence of *n*-hexane is shown. W3110 cells carrying pBSII and pHA105 were spotted on LBGMg medium. W3110 cells exposed to salicylate were spotted on LBGMg medium containing 2 mM sodium salicylate. The spots contained approximately 10 (column 1), 10^2 (column 2), 10^3 (column 3), 10^4 (column 4), and 10^5 (column 5) cells.

TABLE 3. Effects of *marA*, *robA* and *soxS* on antibiotic resistance

Antibiotic	logP_{ow}	MIC (μg/ml) for W3110 cells carrying:			
		pBSII	pHA105 (*marA*)	pOST41BR (*robA*)	pHc3R (*soxS*)
Novobiocin	3.84	200	800	800	800
Nalidixic acid	1.57	6.3	25	25	50
Chloramphenicol	1.14	6.3	50	50	50
Erythromycin	0.65	200	400	800	800
Ofloxacin	−0.01	0.10	0.39	0.39	0.78
Phosphomycin	−0.26	1.6	12.5	6.3	12.5
Norfloxacin	−0.54	0.10	0.78	0.78	0.78
Enoxacin	−1.29	0.20	0.78	0.78	0.78
Tetracycline	−1.86	3.1	6.3	6.3	6.3
Kasugamycin[a]	−3.75	400	400	400	400
Gentamicin[a]	−4.08	3.1	3.1	3.1	3.1
Kanamycin	−7.77	6.3	6.3	6.3	6.3
Neomycin	−9.03	12.5	12.5	12.5	12.5

[a] *E. coli* JA300 was used for the MIC assay.

conserved region of the *marR* gene. We believe that the *marR08* gene loses its function as a *mar* repressor and derepresses the *mar* operon. We observed that overexpression of the *marR08* gene conferred cyclohexane tolerance on JA300, which has the wild-type *marR* gene on its chromosome. On the other hand, strain OST3408 carrying the high-copy-number wild-type *marR* gene (pHA104; Table 2) lost cyclohexane tolerance. MarR is considered to form concentration-dependent oligomeric structures to bind the *marO* operator (37). This result suggests that MarR08 forms heterologous, less functional multimers with wild-type MarR, but excess wild-type MarR can form a functional structure in spite of the existence of MarR08. Several *E. coli* strains were provided the cyclohexane-tolerant phenotype by introducing the *mar* locus of OST3408 utilizing P1*kc* transduction selected with the *zde-234*::Tn*10* marker. Introduction of this locus by P1 transduction may be a useful method for improving organic solvent tolerance of *E. coli* strains.

We observed that exposure to salicylate increases the organic solvent tolerance of *E. coli* cells. This result is expected and reasonable. Salicylate appears to induce the *mar* operon by binding to MarR and inhibiting complex formation with the *marO* operator (27). But it is not easy to optimize the condition of salicylate exposure, because salicylate itself is toxic to microorganisms. Cohen et al. reported that exposure of *E. coli* cells to 5 mM sodium salicylate increased resistance to various antibiotics (11). We observed that exposure to 5 mM sodium salicylate slightly inhibited growth of W3110 cells and decreased organic solvent tolerance. Exposure to 0.5 mM salicylate had no effect on the solvent tolerance of *E. coli* W3110 (data not shown). We also observed that treatment with redox-cycling reagent, which induces the *sox* regulon genes, increases organic solvent tolerance at the same level of overexpression of the *soxS* gene in several *E. coli* strains (data not shown). Addition of these chemical compounds is a simple and convenient method to improve the organic solvent tolerance of *E. coli* strains. We expect that this method may be utilized for industrial *E. coli* strains.

Derepression of the *mar* operon causes an increase in MarA, a transcriptional activator protein, and elevates expression of the *mar* regulon genes (15). We observed that overexpression of the *marA* gene raised the organic solvent tolerance of *E. coli* strains. We conclude that induction of *mar* regulon genes increases organic solvent tolerance of *E. coli* cells.

We previously reported that overexpression of the *soxS* and *robA* genes increased organic solvent tolerance in *E. coli* (32, 33). Some of the *mar* regulon genes overlap members of the *soxRS* regulon (21). Overexpression of the Rob protein increases resistance to multiple antibiotics, including chloramphenicol, tetracycline, nalidixic acid, and puromycin (6, 33). We propose that overexpression of the *marA*, *robA*, and *soxS* genes raises the organic solvent tolerance of *E. coli* cells by the common response to general stress, such as superoxide radicals and antibiotic agents.

Little is known about mechanisms of organic solvent tolerance in bacterial cells. An energy-dependent export system may be responsible for the resistance of *P. putida* S12 to toluene (20). The *mar* locus of *E. coli* may positively influence an active efflux of chloramphenicol (29). Overexpression of the alkylhydroperoxide reductase gene increases organic solvent resistance in *E. coli* (13). There is a possibility that induction of the *mar* regulon activates such efflux pumps or hydroperoxide reductases.

We observed that overexpression of the stress response genes increased resistance to a broad spectrum of antibiotics but not to several highly hydrophilic antibiotics, including kasugamycin, gentamicin, kanamycin, and neomycin. All these drugs are members of the basic oligosaccharide group. It is suggested that the general stress response system does not interact with basic oligosaccharide antibiotics or highly hydrophilic compounds. There is the possibility that activation of the stress response system by MarA, SoxS, and Rob activators nonspecifically increases the resistance to hydrophobic chemical compounds, including organic solvents.

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